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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Epstein-Barr Virus (EBV) contributes causally to specific lymphomas and carcinomas in people. A recent retrospective survey detected EBV DNA in 51 of 100 consecutive breast carcinoma biopsies by PCR, in 3 of 30 normal adjacent biopsies, and a viral protein, EBNA-1, in a fraction of tumor cells in 9 of 9 EBV-positive biopsies assayed immunohistochemically (Bonnet *et al.*, J. Natl. Cancer Inst. 91: 1376-1381, 1999). These data reveal a statistically meaningful association of EBV with breast carcinoma which we shall examine further to confirm or refute. The EBV in positive biopsies is either in the carcinoma cells or in surrounding normal cells such as B-lymphoid cells, a normal site for EBV's latent infection. To assess this association we shall use fixed blocks of tissue for which pairs of one normal adjacent tissue and one breast carcinoma are available. The percent of carcinoma cells present in fixed blocks will be estimated to be less than 1%, from 1% to 10%, or greater than 10%, and the amount of EBV present per cell in all samples determined by multiplex PCR and glass microarrays to measure EBV DNA from known levels of isolated sample DNAs.

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Introduction

We want to test the possible association of Epstein-Barr Virus (EBV) with breast carcinomas. In our application we proposed to use quantitative competitive PCR to detect any EBV DNA present in fixed blocks of tissue from patients taken more than ten years ago. During this funding period we have recognized that we could screen fixed biopsies more generally if we could develop glass microarrays with multiple probes for EBV DNA and use them to detect the presence of EBV DNA in the fixed samples of breast carcinomas. We have succeeded in developing this approach.

Body

Making glass microarrays with EBV probes

In order to maximize the detection of EBV, 22 regions of its DNA have been amplified by PCR to yield products of 300-500 base pairs in length. These products were precipitated with isopropanol and resuspended in water for quantitation. The products were dried and resuspended in an equal volume of water and DMSO at a final concentration of 125 nanograms per microliter and transferred to a 384-well plate for printing. Flat-headed pins from the Total Array System slide printer (BioRobotics) were used to transfer approximately ten picoliters of DNA solution from each well to the glass slides. Glass slides were purchased from Corning and are coated with gamma amino propyl silane for a DNA-immobilizing surface. Printed slides were baked at 85 degrees Celsius for four hours to fix the DNA to the slides. A sample slide was treated with SYBR Green (Molecular Probes) at a ratio of 1:10,000 SYBR Green to 1X TBE and scanned in an Avalanche Array Scanner (Molecular Dynamics) in order to determine uniformity of DNA delivery to the slide.

Although the 22 regions of EBV DNA have all been tested as probes we now use only nine of them as targets (see Table 1). These targets are generated by multiplex PCR of DNAs isolated from either cell lines used as controls or from the fixed tumor samples.

Table 1: Primers for Multiplex PCR

Position in EBV genome	Gene	Length of Product
2010-2483	BNRF1	473bp
24955-25370	BWRF1	438bp
66717-67107	BPLF1	413bp
72748-73185	BOLF1	457bp
79180-79065	BaRF1	445bp
97246-97700	EBNA3b	476bp
145585-145998	BVRF1	437bp
149250-149648	BdRF1	420bp
159507-159848	BALF3	362bp

Sensitivity of detection of EBV DNA using glass microarrays

In order to determine the sensitivity of detecting EBV DNA with microarrays, we first examined our ability to detect EBV DNA in the EBV-positive Burkitt's lymphoma cell line, Raji. 100 nanograms of genomic DNA isolated from Raji cells was used as template in a multiplex PCR reaction containing the nine sets of EBV specific primers. These PCR reactions were purified via QIAGEN PCR Purification Kit and quantitated via a spectrophotometer. The purified PCR product was then used as template for labeling with Cy3-dUTP (Amersham Pharmacia Biotech). Approximately 250 nanograms of the amplified DNAs were combined with ten picomoles of each of the EBV specific primers used in the initial PCR reaction. The DNA was denatured and the primers allowed to anneal. To this mix dNTPs, buffer, Cy3-dUTP and Klenow polymerase were added and incubated at 37 degrees Celsius for three hours. The labeled product was then purified over a Sephadex G-50 column (Roche) and analyzed via spectrophotometry for the efficiency of Cy3-dUTP incorporation. We found that an incorporation efficiency of at least one labeled dye per 60 nucleotides is required for detection of the target DNA on the glass slides. Samples were then dried, resuspended in hybridization buffer, denatured and applied directly to the printed slide. A coverslip was placed on top of the sample and the slide was incubated in a water bath at 55 degrees Celsius overnight. Hybridized slides were then washed and scanned.

To determine the limit of detection of EBV DNA in this assay, genomic DNA from the EBV-positive Raji cells was diluted into genomic DNA from EBV-negative BJAB cells at various ratios. These diluted EBV samples were then used as templates in the multiplex PCR reaction. One-hundred nanograms of DNA from dilutions of 1:100, 1:1000 and 1:3000 Raji:BJAB genomic DNA were used as templates in multiplex reactions, labeled and hybridized to slides. Signals were detected for the 1:100 and 1:1000 dilutions but not the 1:3000 dilution. One hundred nanograms of 1:1000 Raji:BJAB cells is approximately fifteen EBV-positive cell equivalents within a total of 15,000 cells. These conditions provide the sensitivity we require to screen tumor samples.

We now have 29 matched samples of fixed, paraffin-embedded breast cancer specimens. Each of these tumors is matched with normal tissue from the same patient. We now isolate DNA from these samples by adding 1mL of xylene to each 20 micron section to de-paraffinize the sample and washing with 100% ethanol. The sample is then resuspended in 100ul K buffer (50 mM KCl, 10mM tris-HCl, 2.5mM MgC12, 100ug/mL gelatin, 0.45% IGEPAL, 0.45% Tween 20, 6ug proteinase K) and incubated at 55 degrees Celsius overnight. Proteinase K digestion is followed by multiple phenol:chloroform extractions. Since matched normal tissues will be largely composed of adipose tissue and contain fewer cells than tumor tissue, we shall employ realtime quantitative PCR in order to measure the amount of DNA recovered from each sample. A standardized amount of DNA (dependent on recovery) will be used as template for the multiplex PCR described previously.

Key Research Accomplishments

- Development of spotted slide arrays containing Epstein-Barr Virus DNA as a novel method to detect viral DNA in tumor samples
- Application of multiplex PCR for amplification of viral targets and the ability to label these products with Cy3
- Based on work supported by this grant we applied for and received NCI funding to continue to screen breast cancers and other human cancers for the presence of EBV as well as other human tumor viruses.

Reportable Outcomes

On the basis of the work funded by this grant, we participated in an NCI grant application, which was subsequently funded and will support the development of technology to detect novel virus/tumor associations.

Conclusions

Our development of detecting EBV target DNAs through a combination of multiplex PCR to prepare a complex target composed of nine regions of EBV DNA and their detection with DNA probes printed on glass microarrays has provided a sensitive, facile means to detect specific DNAs in fixed tumor samples. We have also mastered the efficient isolation of DNA from 20 micron sections of fixed tumor samples. We have established a collaboration with Dr. Andreas Friedl of the Department of Pathology and Laboratory Medicine who is reading sections adjacent to those from which we extract DNA to estimate the fraction of tumor cells present in the samples we analyze. We are now screening these samples for the presence of EBV DNA. If we fail to detect EBV DNA in these tumor samples, we shall extend our screen to include other known human tumor viruses. Our glass microarrays can accommodate at least 4000 spots so that we shall be able to add probes from multiple regions of all known human DNA tumor viruses to our arrays to screen for any of them that may be associated with breast cancer.

References

None.

Appendices

None.

Personnel Receiving Pay from the Research Effort

Jacqueline G. Perrigoue, Associate Research Specialist